PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68	A1	(11) International Publication Number: WO 99/25872
		(43) International Publication Date: 27 May 1999 (27.05.99)

(21) International Application Number: PCT/GB98/03541

(22) International Filing Date: 19 November 1998 (19.11.98)

(30) Priority Data:
9724480.0 19 November 1997 (19.11.97) GB

(71) Applicant: HEXAGEN TECHNOLOGY LIMITED [GB/GB]; 214 Cambridge Science Park, Milton Road, Cambridge CB4 4WA (GB).

(72) Inventors: FOSTER, Jamie, William; Hexagen Technology Limited, 214 Cambridge Science Park, Milton Road, Cambridge CB4 4WA (GB). SCHAFER, Alan, John; Hexagen Technology Limited, 214 Cambridge Science Park, Milton Road, Cambridge CB4 4WA (GB).

(74) Agent: HALLYBONE, Huw, George; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).

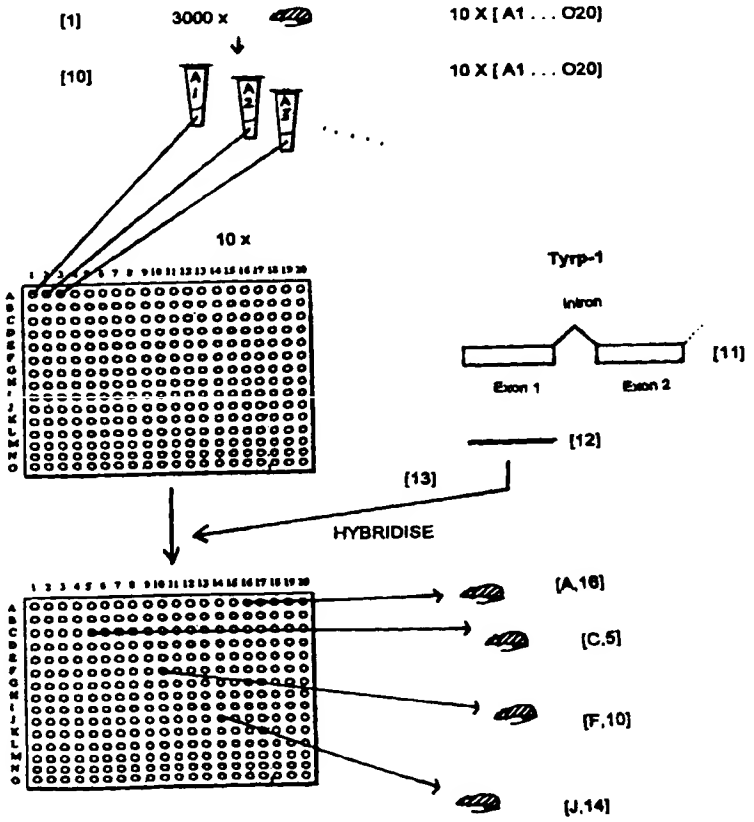
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: HETEROZYGOTE SCREENING PROCESS

(57) Abstract

In order to facilitate the screening of an organism, or a population of organisms, carrying heterozygous mutations for identifying the presence of a mutation in a gene of interest, a process is provided which utilises mismatch binding proteins, such as MutS. The process comprises the steps of: obtaining a nucleic acid sample from the organism; denaturing any double stranded nucleic acid present in the sample; allowing the nucleic acid to anneal; and removing homoduplexes from the annealed sample. A positive signal from a probe specific for the gene of interest indicates that the organism carries a heterozygous mutation in said gene.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

HETEROZYGOTE SCREENING PROCESS

This invention relates to the screening of organisms carrying heterozygotic mutations.

Background to the invention

In order to study the effects of genetic mutation, it has been proposed to use an in-bred population whose individuals carry heterozygotic mutations (*eg.* patent application WO97/44485; Schafer & Hawkins (1998) *Nature Biotechnology* 16:33-39). This population can be screened at a genomic level (*eg.* using single-strand conformation polymorphism) in order to determine those individuals which carry a mutation in a given gene (*ie.* those which carry one mutant copy of a gene of interest and one normal copy), even though the effect of the mutation may not be manifested as a phenotype (*eg.* a recessive mutation). These individuals can then be bred to create organisms for functional studies which carry homozygotic mutant alleles.

To be most useful, the heterozygotic mutant population should satisfy two criteria. Firstly, mutations across the entire genome should be represented. This ensures that the population will contain an individual carrying a mutation for any given gene. Secondly, for any given gene there should be more than one mutant-carrying individual. This ensures that a diverse range of mutations are available.

These goals can be achieved in two ways: each individual can carry a large number of mutations and/or the population can be large.

A large number of mutations per individual is, however, undesirable for three principal reasons. Firstly, too many mutations per individual increases the likelihood of lethal consequences. Secondly, interactions between mutated genes can complicate matters. Thirdly, in order to draw valid conclusions about the effects of a mutation in a given gene it is necessary to ensure that mutations in other genes are not contributing towards the overall phenotype. This means that mutations in other genes must be removed by breeding and the burden of doing so increases with the number of mutations carried by an individual.

In order to reduce the number of mutations per individual whilst maintaining the overall number of mutations represented in a population, it is therefore necessary to increase population size. As population size increases, however, the burden of maintaining the population also increases (*eg.* a colony of 10,000 mice). Furthermore, the burden of screening

the population also increases, in terms of both time and expense.

Whilst the former problem can be reduced by removing the need to maintain a living population whilst keeping the ability to screen the population and reproduce an individual of interest (*eg.* patent application PCT/GB98/01945, which uses two samples per individual: one for genetic screening and the other for reproductive purposes), the burden of screening a large population remains.

It is therefore an object of the invention to facilitate the screening of a population carrying heterozygous mutations in order to identify those members of the population which carry a mutation in a gene of interest.

Where two copies of a gene differ slightly in sequence (*eg.* point mutations), their nucleic acid strands can form heteroduplexes (*ie.* double stranded nucleic acid containing non-base-paired mismatches). Proteins are available which bind heteroduplexes in preference to homoduplexes and these are collectively known as "mismatch binding proteins" (MBPs) *eg.* bacterial protein MutS, which binds to mismatches in otherwise complementary nucleic acid duplexes.

WO95/12689 suggests using immobilised MBPs for detecting heterozygosity in a sample from a single organism. This involves incubating amplified DNA with immobilised MBP and then investigating the bound heteroduplexes with labelled probe DNA [see also Wagner *et al.* (1995) *Nucleic Acids Research* 23:3944-3948].

Description of the invention

According to the invention, there is provided a process for preparing a sample of screening material from an organism carrying heterozygous mutations, comprising the steps of:

- obtaining a nucleic acid sample from the organism;
- denaturing any double stranded nucleic acid present in the sample;
- allowing the nucleic acid to anneal; and
- removing homoduplexes from the annealed sample.

At heterozygotic positions in the genome (*ie.* one mutant allele and one normal allele), nucleic acid which is denatured and re-annealed can hybridise to form heteroduplexes, whereas homozygous alleles (*ie.* neither sequence mutated) will form homoduplexes. Any heteroduplex will thus be made up of a normal strand and a mutated strand. If homoduplexes

are removed (or, put another way, if heteroduplexes are enriched), the sample will represent those alleles in the original sample where one copy was a mutant form.

If the organism carries one mutation in every 1000 genes, for instance, this has the effect of greatly reducing the complexity of each sample (*eg.* from 100,000 different sequences to 100 different sequences).

Furthermore, whereas the original sample represents both homozygous and heterozygous alleles, the final sample (*ie.* the screening material) is enriched for heterozygous alleles. Unless two copies of a gene differ (*eg.* wild-type and mutant), no heteroduplex will form. Whilst a probe for a gene of interest may hybridise with a sample before homoduplex removal, therefore, it will only hybridise with the sample after homoduplex removal if the sample contained two different copies of the gene of interest. A positive signal from the probe therefore indicates that the organism from which the sample was derived carried a mutant copy of the gene of interest. The invention therefore provides a method for determining whether an organism carries a heterozygotic mutation in a gene of interest.

The sample is also suitable "genetic screening material" for use according to PCT/GB98/01945.

The nucleic acid sample will typically comprise DNA but may alternatively comprise RNA. DNA is preferably in the form of cDNA, but genomic DNA may also be used. Nucleic acid may be fragmented prior to the annealing or removal steps, preferably such that the fragments are 200-400bp in size. This can typically be achieved by using a restriction enzyme, although a plurality of restriction enzymes are preferably used to generate overlapping fragments.

The nucleic acid sample may be derived from various sources. For instance, cDNA could be prepared from an individual's major organs and combined *eg.* to compensate for differential expression patterns. Alternatively, the sample could be derived from a single organ or cell-type.

In order to provide sufficient material for screening, rather than preparing nucleic acid from a large number of cells, nucleic acid could be amplified [*eg.* see Abramson & Myers (1993) *Curr Opin Biotech* 4:41-47; Zhang *et al.* (1992) *PNAS* 89:5847-51]. If amplification is used, however, this preferably occurs after heteroduplex formation (*ie.* after the sample is denatured and annealed) and after heteroduplex removal, due to the infidelity of polymerase replication used during amplification. Any errors introduced in this way can result in the formation of a heteroduplex which could be misinterpreted as being due to the presence of a heterozygotic

mutation, or which may reduce detection sensitivity by increasing background noise.

If amplification is used before heteroduplex formation, therefore, the amplification products are preferably treated to remove amplification errors (*eg.* see WO95/12689).

Stringent annealing conditions can be used to eliminate or reduce hybridisation between non-allelic loci. During annealing, mutant sequences can hybridise with normal sequences to form heteroduplexes although, of course, re-formation of homoduplexes can also occur between identical sequences (whether mutant or normal).

The degree of homoduplex removal should be sufficient so that the binding of a probe specific for a sequence of interest which was homozygous in the organism from which the sample is derived is quantitatively reduced. This will typically mean that the abundance of heteroduplexes relative to homoduplexes will be increased by at least two-fold, preferably by at least five-fold, and more preferably by at least ten-fold or greater (*eg.* 100-fold).

Homoduplexes may be removed using any reagent which binds to heteroduplexes in preference to homoduplexes (or *vice versa*) and which does not chemically modify or cleave its bound nucleic acid. MBPs are preferred, such as MutS, along with its derivatives and homologues. The MBP should be immobilised in some way (*eg.* WO95/12689). The MBP could, for instance, be attached to a solid support. Suitable solid supports include cellulose, polystyrene, dextran, and nitrocellulose. These may be used in any suitable form, including beads (which may be magnetic), membranes, or columns. The immobilised MBP is able to bind heteroduplexes and retain them on the solid support, whilst homoduplexes are not retained. The bound nucleic acid may be eluted from the MBP [*eg.* Jolly *et al.* (1997) *Nucleic Acids Research* 25:1913-1919], thus providing a sample which has been enriched for the presence of heteroduplexes *ie.* homoduplexes have been removed. If amplification is used, this may commence before or after elution. If amplification begins before elution, the amplification process will typically result in dissociation of the MBP/heteroduplex complex anyway (*eg.* during the denaturing step in PCR).

The nucleic acid sample may be derived from any diploid organism. Preferably the organism is an animal, such as an insect, and preferably the animal is a vertebrate, such as a fish or a mammal. Preferred mammals are rodents and humans.

An organism "carrying heterozygotic mutations" can conveniently be prepared by mating two

members of an in-bred (and therefore genetically homogenous) population, one of whose germ cells have been exposed to mutagenic conditions. The gametes of this mutagenised organism will thus carry mutations, and the organism will transmit chromosomes carrying random mutations to its progeny. The other organism, which has not been exposed to mutagen, will transmit normal chromosomes. The offspring of such a mating will receive one set of chromosomes from each parent and they will thus be heterozygous for every locus which was mutated in the mutagenised parent, because they will carry one chromosomes with a mutant allele and one chromosome with a normal allele (*ie.* heterozygous mutations). It is also possible to mutagenise both parent organisms, although this is less preferable. If a low level of mutation is induced, it is highly unlikely that an offspring will inherit two mutant copies (*ie.* homozygous mutations) of any given allele; mutations are still heterozygotic (*ie.* each allele has a mutant and normal copy), but mutations are present in both sets of chromosomes.

Where an organism is said to be "carrying heterozygotic mutations", therefore, it is meant (a) that the genome of the organism has been exposed to mutagenic conditions (b) the mutations which resulted from an exposure are only present in half of the diploid chromosomes (*ie.* heterozygotic - for every mutant allele, there is also a normal allele).

It is thus apparent that an organism "carrying heterozygotic mutations" will typically not have been exposed to mutagenic conditions itself, but its genome (in the form of half of its chromosomes) will have been. The mutagenesis will have happened to an ancestor, but mutations induced in the genome of the ancestor's germ cells will be inherited by the organism "carrying heterozygotic mutations". This also ensures that any mutations detected in the screening material will be passed on by the organism, since the mutations are consistent throughout the organism's gametes and somatic tissue.

The frequency of heterozygotic mutations carried by the organism should thus be characteristic of exposure to mutagenic conditions, rather than reflecting spontaneous or background mutations, which are characterised by their low frequency of occurrence. The heterozygous mutations should thus be present at a frequency substantially above this background frequency. Suitably, the mutation frequency is such that, on average, in every 50000 organisms or fewer (*eg.* one in every 10000 organisms, or one in every 1000 organisms) one mutant copy of a gene occurs which would cause an altered phenotype if bred to homozygosity.

Many suitable methods for inducing mutations are known in the art. These include chemical mutagenesis, radiation, and retroviral or transposon insertion. Fuller accounts of the production of organisms carrying heterozygotic mutations can be found in WO97/44485 and PCT/GB98/01945.

As used herein, "mutation" refers to an alteration in the nucleotide sequence of a gene (including its regulatory sequences) from its wild-type or normal nucleotide sequence which results in the ability to form a heteroduplex with the corresponding normal gene. Thus the term does not include large scale deletions or chromosomal rearrangements, but does include point mutations and small (1-4bp) deletions or insertions. This, of course, includes mutations which may be phenotypically neutral, even when homozygous. The range of these "silent" mutations is diverse but, depending on the gene in question, they might be mutations in non-coding regions, point mutations which do not alter the function of a codon (*eg.* CCU to CCG, or CGG to AGG), and mutations which alter a codon but which ordinarily do not affect the final protein function, such as conservative amino acid substitutions (*eg.* CUU Leu to AUU Ile).

The invention also provides, therefore, a process for preparing a sample of screening material from an organism carrying heterozygous mutations, comprising the steps of:

- obtaining two parent organisms;
- exposing the germ cells of one of the parent organisms to mutagenic conditions;
- mating the two parent organisms to produce an offspring organism carrying heterozygous mutations;
- obtaining a nucleic acid sample from the offspring organism;
- denaturing any double stranded nucleic acid present in the sample;
- allowing the nucleic acid to anneal; and
- removing homoduplexes from the annealed sample.

According to a further aspect of the invention, there is provided a sample of nucleic acid heteroduplexes obtainable by any of these processes.

The invention also provides a process for screening a population of organisms carrying heterozygous mutations in order to identify those members of the population which carry a mutation in a gene of interest, comprising the steps of:

- preparing samples of screening material for the members of the population, as described above; and

– contacting the samples with a probe specific for said gene of interest.

Preferably the population comprises 100 or more organisms (*eg.* 1000, 10000, 100000, or more).

The samples of screening material are preferably arrayed prior to probing. This might take the form, for example, of fixing samples from 10000 individuals in a 100x100 array on a nitrocellulose membrane.

The probe is specific for the gene of interest and is labelled such that hybridisation between the probe and a sample of screening material can be detected. This may be a radioactive label, for instance, or a fluorescent label.

The samples with which the probe hybridises are derived from an organism carrying a mutation in the gene of interest. It will, of course, be appreciated that whilst background or non-specific hybridisation might occur in samples which are not derived from an organism carrying a mutation in the gene of interest, this will be weaker than the hybridisation which occurs with a sample which is derived from an organism carrying a mutation in the gene of interest. The samples of screening material from organisms not carrying a mutation in the gene of interest will, in fact, serve as controls for background or non-specific hybridisation, and hybridisation above the control level is indicative of a positive result.

In an array of 100x100 samples, for instance, hybridisation with a radioactive probe will occur at a low level throughout the array, but a number of samples will hybridise strongly with the probe.

It will be appreciated that the probing step can be adapted for the use of multiple probes. For instance, probes specific for different genes or specific for different regions in the same gene can be used simultaneously. Thus the invention also provides a process for screening a population of organisms carrying heterozygous mutations in order to identify those members of the population which carry mutations in a plurality of genes of interest.

Where multiple probes are utilised, these are preferably differentially labelled (*eg.* differently coloured fluorochromes in each probe).

It will also be appreciated that the population need not be screened at the level of the individual. For example, in a population of 10000 organisms, screening samples from groups of ten individuals could be combined to give 1000 combined screening samples. These are screened in the same way as the 10000 would be screened, but the probing results require

deconvolution in order to determine which of the ten organisms in a positive combined screening sample carry a mutation. This would typically involve screening the ten separate samples which were originally pooled. Whilst combining samples in this way obviously reduces the initial screening effort (*eg.* from 10000 samples to 1000 samples), the sensitivity of the assay is reduced and the requirement for deconvolution arises. The choice of whether to combine and, if so, how many samples to combine (*eg.* 10, 50, 100 *etc.*) therefore depends on the available resources, the size of the population, the degree of homoduplex removal, *etc.* This choice can be made without difficulty by the skilled person.

Brief description of the drawings

Figure 1 shows a scheme for generating mice carrying heterozygous mutations, and **Figure 2** shows how screening material from such mice can be prepared.

Figure 3 shows how a population of these mice can be screened to identify those members of the population which carry a mutation in the first exon of the Tyrp-1 gene. Similarly, **Figure 4** shows the screening of the population for mutations in the first two exons of Tyrp-1 and in the first exon of cKit.

Figure 5 shows how pooling might be used according to the invention.

Examples

Generation of mice carrying heterozygotic mutations

Random mutations were induced in the genome of premeiotic spermatogonia of 300 male mice (strain C3Heb/Fej) using ethylnitrosourea (ENU). Three separate doses of 100 mg/kg body weight ENU were injected interperitoneally, with each injection separated by a one week interval. Approximately one third of the mice were rendered permanently sterile, but after 8-14 weeks the other two thirds were mated with two non-mutagenised females each, producing F1 offspring carrying heterozygous mutations in the genome of their somatic and germ tissue (see Figure 1). About 1000 F1 offspring were generated per week in this way.

Extraction of nucleic acid (Figure 2a)

At 6 weeks old, a 200µl blood sample and a short tail-clipping were taken from 3000 F1 mice [1]. For each sample [2] from each mouse [1], RNA and genomic DNA were extracted

separately using standard protocols (*eg.* Sambrook *et al.*). The RNA was converted to double stranded cDNA with reverse transcriptase.

10μg DNA from each F1 mouse was digested using *Sau3AI* in a total reaction volume of 30μl (manufacturer's recommended conditions). Following digestion, the DNA was precipitated by the addition of 3μl sodium acetate (3.0M) and 80μl ethanol. The DNA was collected at the bottom of the reaction tubes [3] by centrifugation at 15,000*g* for 30 minutes and the ethanol was aspirated and discarded. The nucleic acid was denatured and re-annealed [4] using the phenol enhanced re-association technique (PERT) [Miller & Riblet (1995) Improved phenol emulsion DNA re-association technique (PERT) using thermal cycling. *Nucleic Acids Research* 23:2339-2340; this technique is suitable for reducing hybridisation between non-allelic sequences]. The restriction digested, double-stranded DNA samples were re-suspended in 50μl PERT buffer (1.5M sodium thiocyanate, 120mM sodium phosphate, 10mM EDTA, 8% phenol) and denatured by heating to 100°C for 10 minutes and quickly chilled on ice to form an emulsion in the reaction. The samples were then placed in a programmable thermal cycler and cycled for 24 hours at 65°C (2 minutes) and 37°C (15 minutes). The samples were centrifuged at 15,000*g* for 10 minutes and the upper phenol phase removed and discarded. The DNA was precipitated by adding 5μl sodium acetate (3.0M) and 125μl ethanol. The DNA was collected at the bottom of the reaction tubes by centrifugation at 15,000*g* for 30 minutes and the ethanol was aspirated and discarded. The DNA was re-suspended in 20μl mismatch binding protein reaction buffer (20mM Tris-HCl, pH 7.6, 5mM $MgCl_2$, 0.1mM dithiothreitol, 0.01mM mM EDTA) and 3% BSA.

Each sample [5] was given an identifier so that the mouse from which any particular sample was derived could be traced, and the digested genomic DNA and cDNA samples were stored.

Homoduplex removal (Figure 2b)

Disposable MBP columns [6] were prepared by applying 500ng MutS protein (Amersham UK) to nitrocellulose columns in 50μl MBP reaction buffer. The DNA samples were removed from storage, applied to the columns and incubated at room temperature for 30 minutes to allow heteroduplex DNA molecules to be bound by the MutS MBP. Un-bound homoduplex molecules [9] were removed from the column by five washes [7] of 200μl MBP reaction buffer ("Wash conditions"). Bound heteroduplex DNA molecules were eluted [8] by incubating the column at 37°C for 30 minutes in 50μl elution buffer (100mM Tris-HCl,

150mM NaCl, 100ug/ml proteinase K). Eluted DNA [10] was precipitated by the addition of 5µl sodium acetate (3.0M) and 125µl ethanol and collected at the bottom of the reaction tube by centrifugation at 15,000*g* for 30 minutes. The ethanol was aspirated and the DNA re-suspended in 10µl TE (10mM Tris-HCl, pH 7.5, 1.0mM EDTA).

This eluted heteroduplex DNA [10] can be used as screening material.

The amount of DNA in the blood samples was lower than that in the tail-clippings, thus reducing the quantity of heteroduplex DNA which was recovered from each sample. To compensate for this, the blood-derived samples of MBP-treated screening material were subjected to whole genome amplification [Zhang *et al.* (1992) *supra*] to give an unbiased linear amplification of the complete nucleic acid used as the starting material. A 5µl aliquot from each sample was placed in a reaction tube containing the whole genome amplification reaction components (100mM Tris-HCl, pH 8.9, 150mM KCl, 200µM each [dCTP, dATP, dGTP, dTTP], 40mM random 15-mer, 1.25U *Taq* polymerase) and the samples were placed in a thermal cycling machine and cycled [94°C 1 minute; 37°C 2 minutes; 37°-55°C ramp at 10 seconds/degree; 55°C 4 minutes; 50 cycles], followed by a final incubation at 72°C for 5 minutes.

This amplified DNA can be used as screening material.

Screening the population (Figure 3)

The samples of heteroduplex-enriched screening material were used in order to find F1 mice which carried mutations in genes of interest. Because of the size of the population, it was anticipated that there would be more than one mouse carrying a mutation in any given gene, resulting in an allelic series of mutations spread throughout the coding and non-coding regions. In order to aid studies of the function of a gene, these mutations would preferably affect the function of the protein in different ways.

One gene of interest was the Tyrosine related protein-1 (Tyrp1; Genbank accession number X03687), a melanocyte-specific enzyme involved in melanin synthesis. Recessive mutant alleles in mice cause a brown coat colour, while a dominant allele causes an almost white appearance in black mice. We were interested in studying mutations in the first exon of the *tyrp* gene [Figure 3 – 11]

1µl of each of the 3000 screening samples [10] was arrayed on nylon hybridisation membranes [13] in a grid pattern using a robotic fluid handling device (10 sheets; 300

samples per sheet: A1, A2, . . . , O19, O20). The nylon membranes were placed on absorbent paper soaked in 0.4M NaOH, simultaneously denaturing the screening samples and fixing them to the nylon membranes.

A probe [12] was produced by PCR amplification of the first exon of the gene from an untreated C3Heb/Fej mouse, using a pair of specific primers which were designed using the PCR primer design program Primer-3.0. This DNA was radio-labelled using a multiprime™ kit (Amersham, UK) incorporating ^{32}P-dCTP, and hybridised [13] to the screening material on the nylon membranes.

The membranes were washed under stringent conditions [0.2xSSC, 0.2% SDS, 65°C] to reduce non-specific hybridisation and then they were exposed to X-ray film. Whilst a low level of radioactivity was visible across the whole film (background hybridisation), four dark spots were apparent on one of the autoradiograms, which were indicative of the probe being preferentially retained where there were complementary sequences. These spots were at positions A16, C5, F10 and J14 in the array, indicating that the mice which gave rise to these four samples carry heterozygotic mutations in the first exon of the Tyrp1 gene.

These four mice were selected from the population and are being subjected to phenotypic analysis. This will, of course, only identify phenotypes if the particular mutation is dominant. The mice are therefore being used for breeding to obtain mice carrying homozygous mutations for fuller functional characterisation. As the F1 were heterozygous for mutations, only half of the F2 mice inherit the Tyrp mutation from the parent and so, for the mutations to be carried homozygously, F3 progeny must be produced. Breeding to further generations (F4, F5, *etc.*) will also ensure that the contribution of any other mutations carried by the mice will be removed.

In addition, the exact nature of each of the mutations which were detected was determined by PCR amplification of the first exon of the Tyrp1 gene from the mouse's genome, followed by sequencing on an ABI automated sequencing machine. For each animal, at one position in the sequence there were two sequencing peaks, indicating that two different alleles were present. The four mutations were distributed across the first exon, giving an allelic series of mutations, albeit a small one.

Creating an allelic series of mutations in a mouse using traditional transgenic methods would have been very labour intensive and time consuming. Screening the population of heterozygous mutant mice was far less onerous and gave satisfactory results.

Simultaneously screening different genes (Figure 4)

We were also interested in studying mast cell growth factor (MGF in mice; known as stem cell factor (SCF) in humans).

MGF is the ligand for the c-kit tyrosine kinase receptor (in humans, c-KIT) and is a haematopoetic growth factor critical to growth of several distinct cell lineages. Mutations in MGF, or in c-kit, can affect the ligand/receptor interaction such that signalling is no longer triggered; mutations which have no such effect are also useful, since they can be used to map regions of the protein which are not critical for function. Dominant mutations of the MGF and c-kit genes in mice affect germ cell development, coat colour and hematopoiesis; mutation of human c-KIT can cause piebaldism, a pigmentation defect, and c-KIT mutations have also been found in mast cell leukemias.

Rather than simply repeating the Tyrp experiment with a MGF-specific probe, however, we developed the screening process so that we could identify mice carrying mutations in MGF at the same time as screening to find those carrying mutations in Tyrp.

This involved preparing a number of different probes. Three probes were used: (1) a probe [14] for the first exon of Tyrp (2) a probe [15] for the second exon of Tyrp (3) a probe [16] for the first exon of c-kit.

These probes were prepared as before, but the PCR primers used to prepare the Tyrp probes were labelled with biotin at their 5' ends and the primers used to prepare the cKit probe were labelled at their 5' ends with fluorescein. The labelled probes were mixed together and hybridised (stringent conditions) with another set of ten nylon membranes containing screening material.

The sites of hybridisation of the Tyrp-1 probes were detected using ELISA incorporating anti-biotin horse radish peroxidase (HRP). The sites of hybridisation of the cKit probe were detected using ELISA incorporating anti-fluorescein alkaline phosphatase (AP).

The spots which gave a positive HRP result, indicated by a red colouration, revealed the identity of mice carrying heterozygous mutations within either the first or second exon of the Tyrp-1 gene. The same four mice as previously detected gave positive results, along with two further mice (K8 & M3). These mice were subsequently shown to carry heterozygous mutations in the second exon.

The spots which gave a positive AP result, indicated by a blue colouration, were M5 and O14. Analysis of the mice from which these two samples were derived shows that they carried heterozygous mutations in the first exon of the c-kit gene.

It will, of course, be appreciated that any suitable form of differential labelling could have been used to distinguish between mutations in the different genes. The three pairs of PCR primers could, for instance, have been labelled with differently coloured fluorochromes. Furthermore, by combining this system with a pair of ^{32}P labelled primers for a further gene, for example, four genes could be screened simultaneously *etc.*

Screening with pooling (Figure 5)

Whilst the screening process used above offers significant advantages when compared with traditional methods, it still used ten nylon membranes per experiment. To reduce the effort required to screen the population, samples of screening material were pooled.

Prior to spotting on nylon membranes, samples of screening material were combined from groups of ten mice. This gave 300 combined samples, rather than 3000 individual samples.

These samples were probed as before with the ^{32}P probe [12] for the first exon of the Tyrp gene. Four positive spots were detected [C8, G6, H3, I5]. To deconvolute the pooling, the ten samples which were combined to give sample C8 were then spotted individually on a small nylon membrane.

This was probed and one spot gave a positive signal. The mouse from which this sample was derived turned out to be mouse C5.

Paired samples

In a further series of experiments, another 1500 F1 mice were bred carrying heterozygotic mutations. At 6 weeks of age, however, these mice were sacrificed. Gametes (sperm or ova) and somatic tissue (spleen, kidney, heart and brain) were harvested from each F1 mouse. cDNA was prepared from the somatic tissue, although genomic DNA could have been used instead. The cDNA from the different organs was combined to give a single combined cDNA sample for each F1 mouse. The gametes were stored in labelled cryo-tubes in five 30x10 racks at -196°C and somatic tissue cDNA was stored in similar racks at -70°C. Each position in a gamete rack corresponded to a position in a somatic rack which contained material taken from

the same mouse.

The five somatic tissue cDNA racks were removed from storage, arrayed, and probed with the Tyrp first exon probe in the same way as before. Two positive signals were seen, and the gametes corresponding to these somatic tissue samples were removed from the freezer. The gametes were used to produce F2 offspring for study as described above.

It will, of course, be understood that the invention is described above by way of non-limiting example only and modifications may be made whilst remaining within the scope and spirit of the invention.

CLAIMS

1. A process for preparing a sample of screening material from an organism carrying heterozygous mutations, comprising the steps of:
   - obtaining a nucleic acid sample from the organism;
   - denaturing any double stranded nucleic acid present in the sample;
   - allowing the nucleic acid to anneal; and
   - removing homoduplexes from the annealed sample.

2. A process according to claim 1, wherein said organism is a mouse.

3. A process according to claim 1, further comprising the step of fragmenting the nucleic acid in the sample.

4. A process according to claim 1, wherein the removal of homoduplexes is effected using immobilised MutS protein.

5. A process according to claim 1, initially comprising the steps of:
   - obtaining two parent organisms;
   - exposing the germ cells of one of the parent organisms to mutagenic conditions; and
   - mating the two parent organisms to produce an offspring organism carrying heterozygous mutations;

6. A sample of nucleic acid heteroduplexes obtainable by a process according to any one of claims 1 to 5.

7. A process for screening a population of organisms carrying heterozygous mutations in order to identify those members of the population which carry a mutation in a gene of interest, comprising the steps of:
   - preparing samples of screening material for the members of the population, according to claim 1; and
   - contacting the samples with a probe specific for said gene of interest.

8. A process according to claim 7, wherein said population comprises 100 or more organisms.

9. A process according to claim 7, wherein said samples are arrayed prior to probing.

FIGURE 1

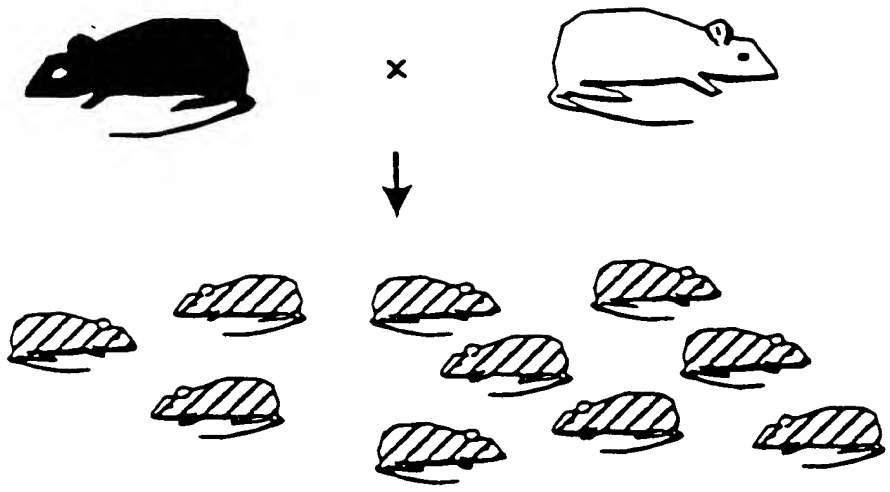


FIGURE 2

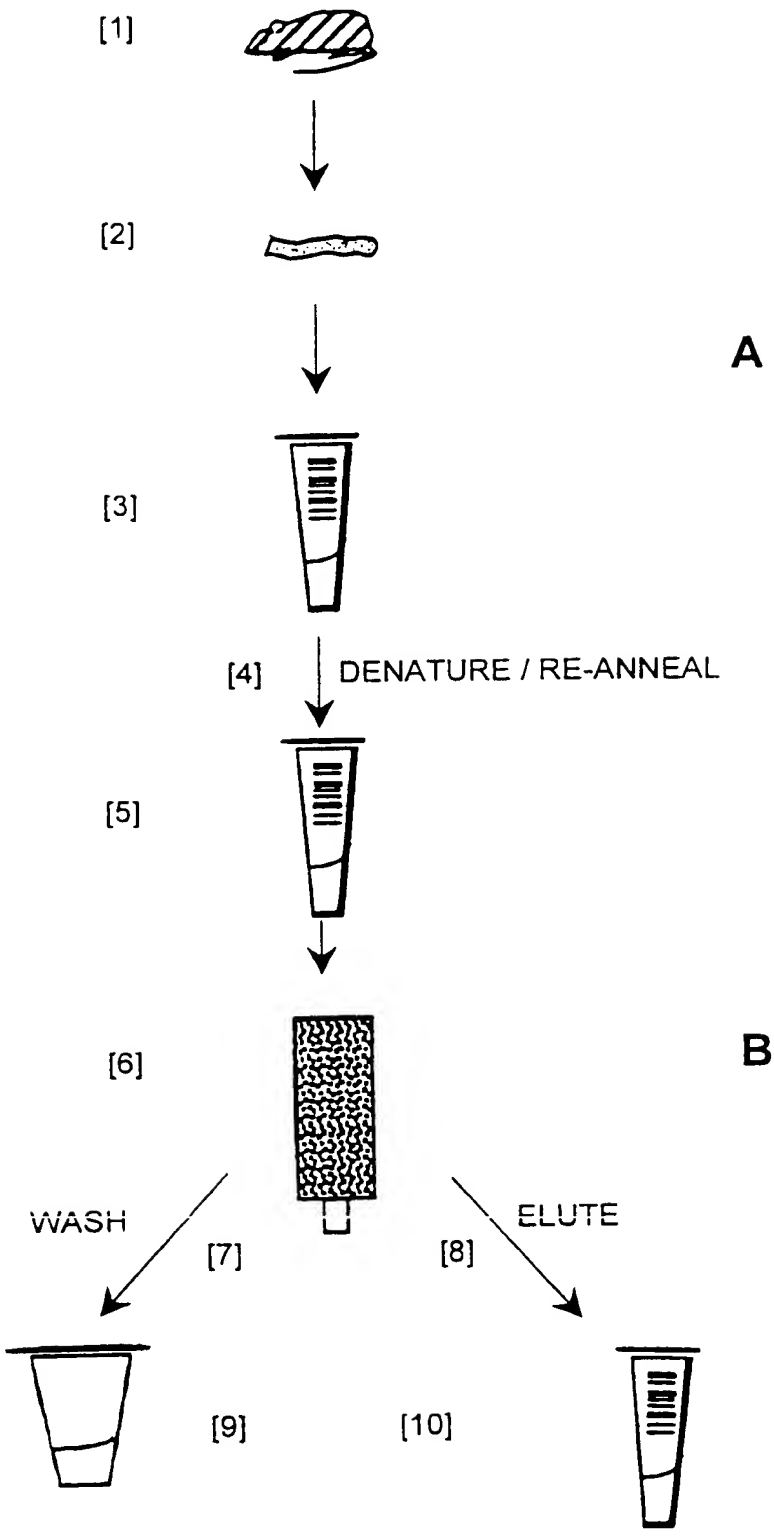


FIGURE 3

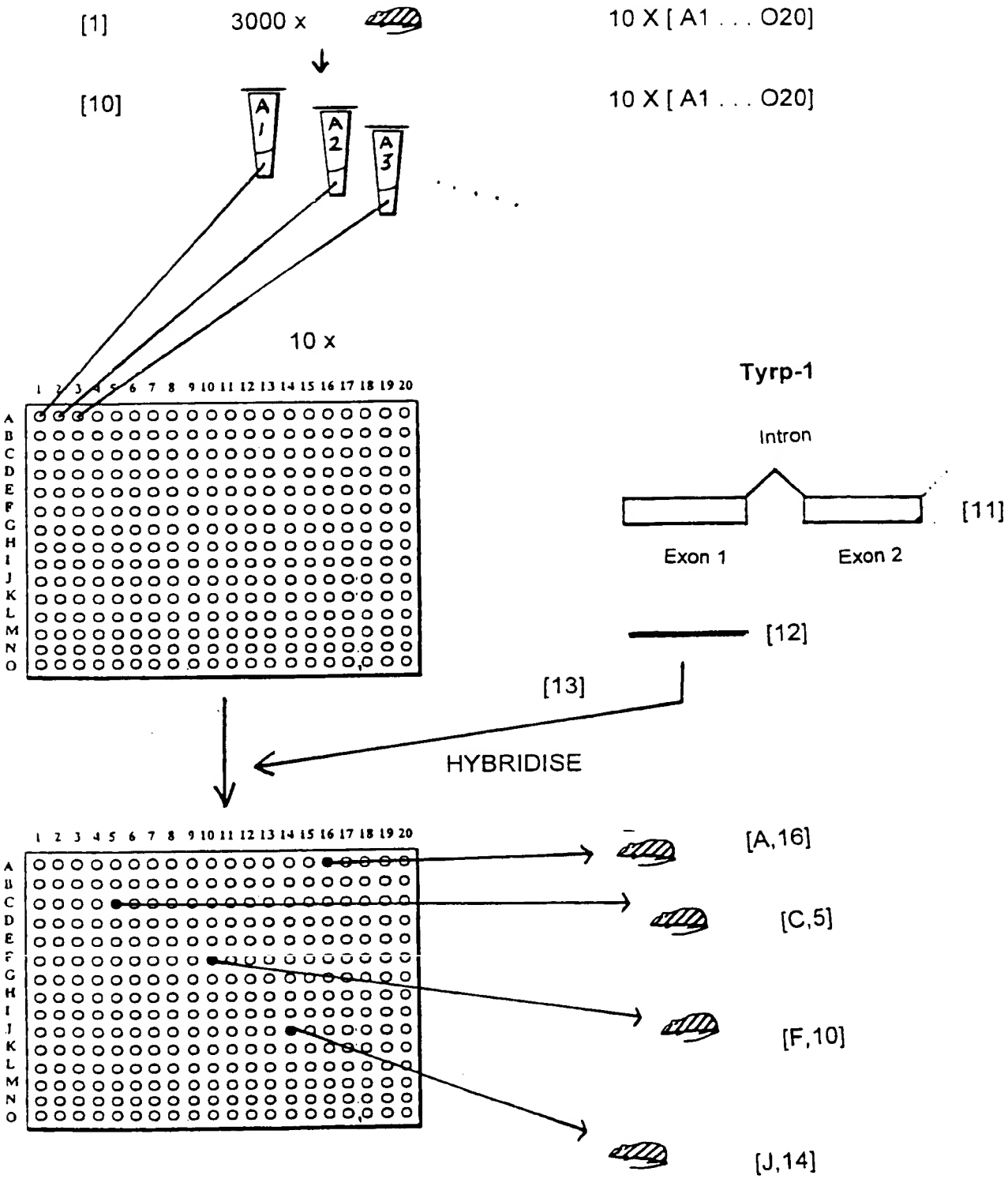

[1]
3000 x
10 X [A1 . . . O20]
[10]
10 X [A1 . . . O20]
A1
A2
A3
10 x
Tyrp-1
Intron
Exon 1
Exon 2
[11]
[12]
[13]
HYBRIDISE
[A,16]
[C,5]
[F,10]
[J,14]

FIGURE 4

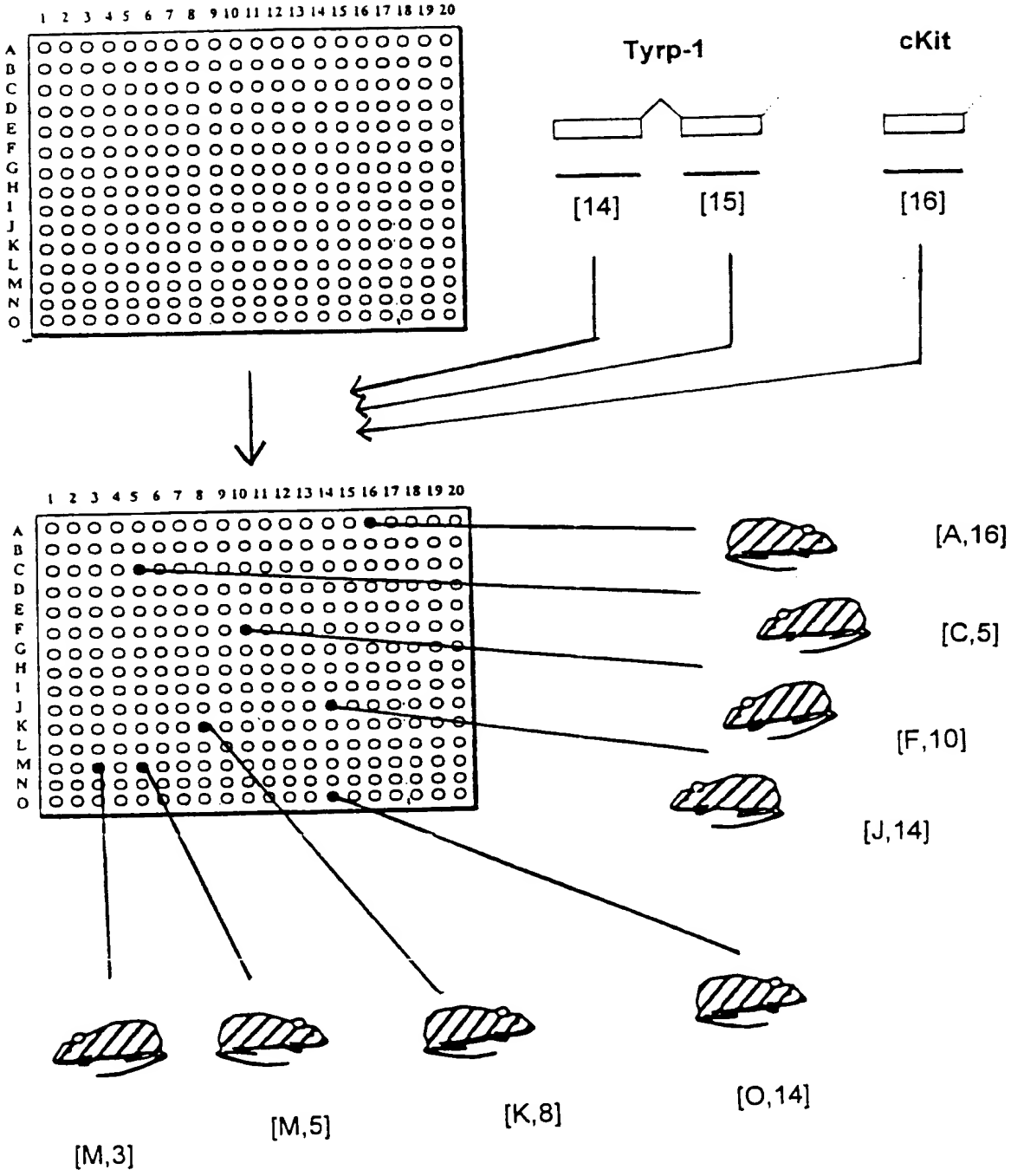
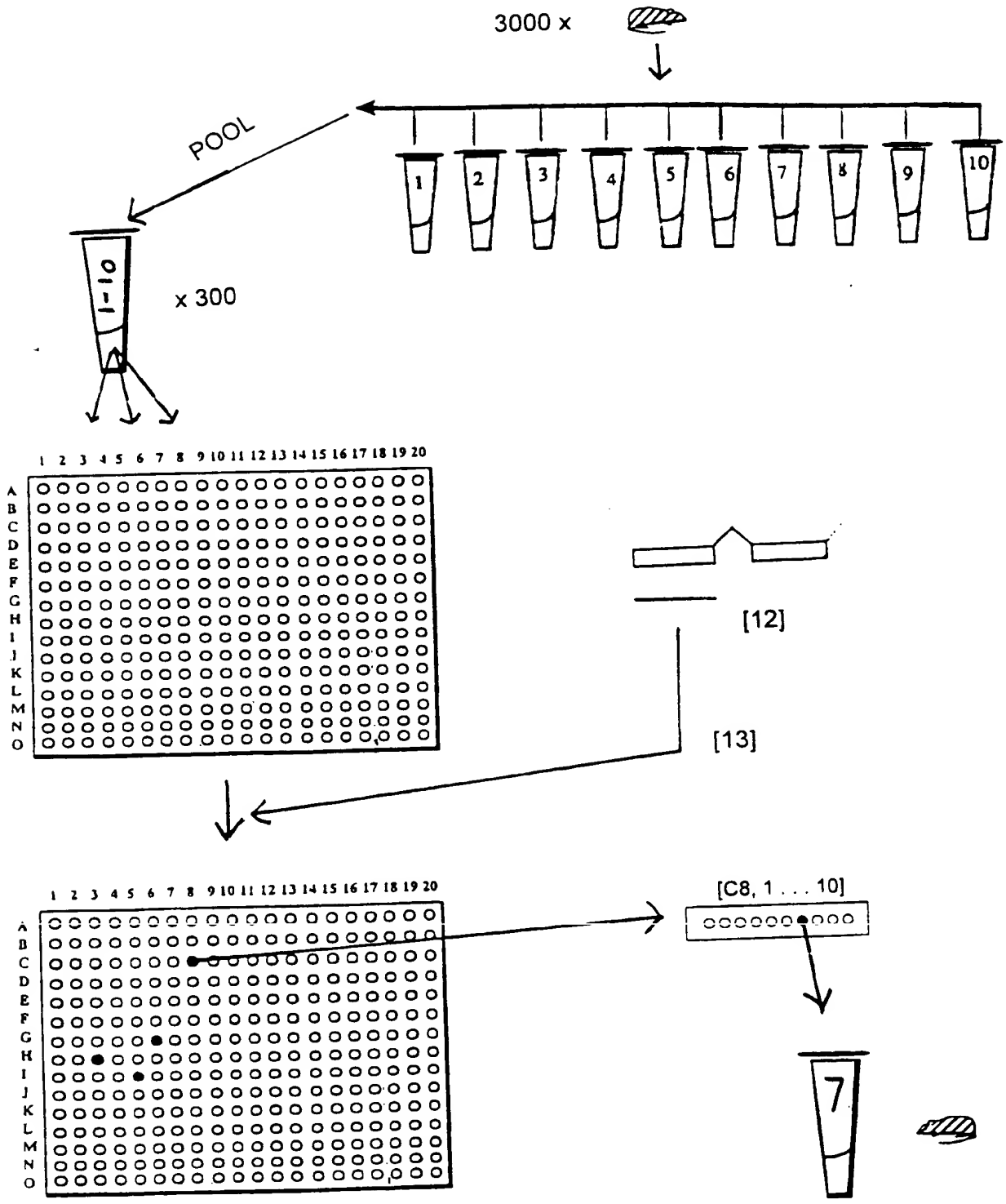

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
A B C D E F G H I J K L M N O
Tyrp-1
cKit
[14]
[15]
[16]
[A,16]
[C,5]
[F,10]
[J,14]
[M,3]
[M,5]
[K,8]
[O,14]

FIGURE 5


3000 x
POOL
1 2 3 4 5 6 7 8 9 10
1-10
x 300
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
A B C D E F G H I J K L M N O
[12]
[13]
[C8, 1 . . . 10]
7

INTERNATIONAL SEARCH REPORT

In tional Application No
PC[redacted] 98/03541

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 22462 A (UNIV LELAND STANFORD JUNIOR) 11 November 1993 See pages 15-16 see the whole document ---	1-9
X	WO 93 22457 A (MASSACHUSETTS INST TECHNOLOGY) 11 November 1993 see the whole document ---	1-9
X	WO 95 12689 A (GENE CHECK INC ;WAGNER ROBERT E JR (US)) 11 May 1995 see the whole document ---	1-9
X	WO 95 12688 A (US BIOCHEMICAL CORP) 11 May 1995 see the whole document --- -/--	1-9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 February 1999	24/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hagenmaier, S

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No PCT/[redacted] 98/03541

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	SMITH AND MODRICH: "MUTATION DETECTION WITH MutH,MutL, AND MutS MISMATCH REPAIR PROTEINS" PNAS, vol. 93, April 1996, pages 4374-4379, XP002093252 see the whole document	1-9
Y	WO 93 02216 A (UPSTATE BIOTECH INC) 4 February 1993 see the whole document	1-9
Y	LISHANSKI ET AL.: "MUTATION DETECTION BY MISMATCH BINDING PROTEIN, MutS, IN AMPLIFIED DNA:APPLICATION TO THE CYSTIC FIBROSIS GENE" PNAS, vol. 91, 1994, pages 2674-2678, XP002093253 see the whole document	1-9
A	WO 96 41002 A (GENZYME CORP) 19 December 1996 see the whole document	1-9
A	WO 95 29258 A (ST JAMES AND SEACROFT UNIVERSI ;TAYLOR GRAHAM ROY (GB)) 2 November 1995 see the whole document	1-9
A	ELLIS L A ET AL: "MUTS BINDING PROTECTS HETERODUPLEX DNA FROM EXONUCLEASE DIGESTION IN VITRO: A SIMPLE METHOD FOR DETECTING MUTATIONS" NUCLEIC ACIDS RESEARCH, vol. 22, no. 13, 11 July 1994, page 2710/2711 XP000606262 see the whole document	1-9
A	ENG AND VIJG: "GENETIC TESTING: THE PROBLEMS AND THE PROMISE" NATURE BIOTECHNOLOGY, vol. 15, May 1997, pages 422-426, XP002093278 see the whole document	1-9
A	MULLINS M C ET AL: "LARGE-SCALE MUTAGENESIS IN THE ZEBRAFISH: IN SEARCH OF GENES CONTROLLING DEVELOPMENT IN A VERTEBRATE" CURRENT BIOLOGY, vol. 4, no. 3, 3 March 1994, pages 189-202, XP002042326 see the whole document	1-9

-/--

1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/03541

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 97 44485 A (HEXAGEN TECHNOLOGY LIMITED) 27 November 1997 cited in the application see the whole document	1-9
P,X	US 5 750 335 A (GIFFORD DAVID K) 12 May 1998 see the whole document	1-9

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/03541

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9322462 A	11-11-1993	US 5376526 A	27-12-1994
		AU 4369593 A	29-11-1993
		CA 2132968 A	11-11-1993
		EP 0672182 A	20-09-1995
		JP 8500723 T	30-01-1994
WO 9322457 A	11-11-1993	US 5750335 A	12-05-1998
WO 9512689 A	11-05-1995	AU 700959 B	14-01-1999
		AU 1050895 A	23-05-1995
		CA 2175222 A	11-05-1995
		EP 0733126 A	25-09-1996
		JP 9504699 T	13-05-1997
WO 9512688 A	11-05-1995	US 5556750 A	17-09-1996
		US 5459039 A	17-10-1995
		US 5861482 A	19-01-1999
		US 5679522 A	21-10-1997
		US 5702894 A	30-12-1997
		US 5858754 A	12-01-1999
		CA 2175490 A	11-05-1995
		EP 0788555 A	13-08-1997
		JP 9504437 T	06-05-1997
WO 9302216 A	04-02-1993	AT 151114 T	15-04-1997
		AU 664381 B	16-11-1995
		AU 2415992 A	23-02-1993
		CA 2113716 A	04-02-1993
		DE 69218776 D	07-05-1997
		DE 69218776 T	27-11-1997
		EP 0596028 A	11-05-1994
		IL 102550 A	18-03-1997
		JP 7500493 T	19-01-1995
WO 9641002 A	19-12-1996	US 5571676 A	05-11-1996
		US 5707806 A	13-01-1998
		AU 5981396 A	30-12-1996
		CA 2219933 A	19-12-1996
		EP 0832283 A	01-04-1998
WO 9529258 A	02-11-1995	AU 2315195 A	16-11-1995
		EP 0758402 A	19-02-1997
WO 9744485 A	27-11-1997	AU 2905497 A	09-12-1997
US 5750335 A	12-05-1998	WO 9322457 A	11-11-1993